THIRTEEN-WEEK NOSE-ONLY INHALATION TOXICITY STUDY OF ROOM AGED CIGARETTE SIDESTREAM SMOKE AND DIESEL ENGINE EXHAUST IN MICE

SPONSOR:
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CONTRACT LABORATORY:
Battelle, Pacific Northwest Laboratories (BNW)
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Protocol Date: _____

I. STUDY TITLE

Thirteen-Week Nose-Only Inhalation Toxicity Study of Room Aged Cigarette Sidestream Smoke and Diesel Engine Exhaust in Mice

II. PROJECT OBJECTIVE

Objectives of this study are to determine the toxicity of RASS and DEE administered by nose-only inhalation to male and female mice 6 hours per day, 5 days per week, for thirteen weeks to evaluate the system, to work out technical difficulties, and to establish laboratory procedures. The study will include groups of mice to be used for studies of potential carcinogenicity mechanisms. The study will also provide data on an additional group which will be allowed to recover for 13 weeks. Results of this study will be used to validate the doses of RASS for a 2-year carcinogenicity bioassay in mice and to select the dose of DEE to be used as the positive control in that study.

III. STUDY SCHEDULE

A. Arrival - week of

B. Start of exposure -

C. Last day of exposure -

IV. PROJECT NUMBER

V. PROTOCOL DATE

VI. SPONSOR

Philip Morris
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VII. SPONSOR'S REPRESENTATIVE

George J. Patskan, PhD

VIII. TESTING FACILITY

Battelle, Pacific Northwest Laboratories (BNW)
Toxicology Department K4-10
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IX. STUDY DIRECTOR AND PRINCIPAL INVESTIGATOR

Earl W. Morgan, DVM, ACVPM, DABT

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X. TEST ARTICLE DESCRIPTION

A. Identification

1. Room Aged Sidestream Smoke (RASS)

RASS will be obtained by aging sidestream smoke (SS) for 0.5 hours (mean age) in a room. SS will be generated from the standard reference filter cigarette, 1R4F, smoked in basic conformity with ISO standards 3402, 4387, and 3308 (1991). The cigarettes will be smoked by a 30-port smoking machine (CH Technologies, Westwood, NJ).

B. Lot Number

C. How Supplied

D. Storage Conditions

Cigarettes will be stored in a freezer at -16 ± 2°C. They will be preconditioned for at least 48 hours at room conditions (22 ± 2°C and 60 ± 10% RH) prior to smoking. Distribution and handling during the course of the study will be conducted in such a manner that proper identification will be maintained and contamination avoided.

E. Source

Philip Morris

XI. POSITIVE CONTROL DESCRIPTION

A. Diesel Engine Exhaust

The DEE will be generated from a light-duty pickup truck engine, mounted on a shock mount engine stand attached to a dynamometer system. The dynamometer will be programmed to simulate the EPA Urban Dynamometer Driving Schedule (40CFR Ch. 1, Pt. 86, App I).

B. Storage Condition

Diesel fuel will be stored in tank external to the building with a small day tank in the generator room.

C. Source

XII. TEST ATMOSPHERE

On-line monitoring of the mass concentration of RASS and DEE will be accomplished by two Tapered Element Oscillating Microbalance Mass Monitors (TEOM 1400, Rupprect & Patashnick Co., Inc., Albany, NY). The TOEMs will sample RASS and DEE aerosols from a representative exposure port in the exposure unit. Exposure concentrations will be controlled to within ±20 % of target concentration by adjustment of the exposure unit dilution airflow.

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A real time aerosol monitor (RAM; MIE, Inc., Bedford, MA) will be used to monitor the concentration of RASS as it enters the distribution line from the aging room. Aerosol concentrations will be monitored with the RAM twice per hour over the duration of the daily generation period. The relative response of the RAM to the aerosol concentration (volts per unit concentration) will be determined prior to the start of exposure. RAM response will be evaluated by comparing the measured RAM voltage to the aerosol concentration of RASS determined from independent filter samples.

The total particulate mass (TPM) of both RASS and DEE in the test atmosphere during the exposure will be determined from duplicate Cambridge filter samples from both the distribution line at the exit from the aging room and two exposure ports from each exposure unit collected over the duration of the generation period. These filters will be analyzed gravimetrically and/or by UV analysis. Filter sample extracts will be analyzed as soon as feasible following collection and preparation.

The buildup and decay of the test article concentration in the exposure unit will be determined prior to start of the study. The time to buildup to 90% of the target concentration (T_{90}) and the time to decay to 10% of the target concentration (T_{10}) will be determined.

The particle size distribution of the aerosol in the exposure unit will be measured prior to the start of exposure and once a month during the exposure phase. Median diameters and geometric standard deviations (GSD) will be reported.

Uniformity of the concentration and particle size distribution of the test article in the nose-only exposure unit will be determined during the prestart phase of the study. Uniformity of test article concentration will be demonstrated by monitoring one port on each level with the TEOM or RAM. Uniformity of particle size distribution will be demonstrated by samples taken at the top, middle and bottom levels of the exposure unit.

XIII. INHALATION EXPOSURE SYSTEM

A. Exposure Room

Rooms 523 and 531, LSL-II Building

B. Exposure Unit

Cannon flow-past nose-only exposure units. Each unit has 104 exposure ports, 90 of which will be used for rat exposures. The remaining ports will be used for sampling or closed off. Each exposure unit will be contained in a rigid clear plastic cabinet to assure no contamination of the room by the test article aerosol. Airflow through the hood will be maintained at approximately 15 cfm to assure proper cooling of the mice in the restraint tubes. The exposure unit can be rotated so that all mice may be easily observed during exposure.

C. Exposure Generator

RASS will be obtained by aging sidestream smoke (SS) for 0.5 hours (mean age) in a 30 m^3 room. SS will be generated from the standard reference filter cigar ette, 1R4F, smoked by a 30-port smoking machine (CH Technologies, Westwood, NJ) in basic conformity with ISO standards 3402, 4387, and 3308 (1991). Mainstream smoke from the smoking machine will be routed through a filter system to remove the majority of the particulate material prior to being routed to the building exhaust. A stainless steel cone will be fitted over the smoking machine to extract the SS which will be mixed with conditioned dilution air (22 ± 2°C and 60 ± 10% RH) before being injected into a smoke aging room by a flow-controlled fan. The

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entire flow from the aging room will be delivered to the exposure rooms via a stainless steel delivery tube. The required flow of RASS will be siphoned from this delivery line by a pressurized-air-controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose only exposure unit. Exhaust from the exposure units will be routed through a filter system to remove the majority of the particulate material prior to being routed to the building exhaust.

DEE will be generated from a light-duty pickup truck engine, mounted on a shock mount engine stand attached to a dynamometer system. The dynamometer will be programmed to simulate the EPA Urban Dynamometer Driving Schedule (40CFR Ch. 1, Pt. 86, App I). A constant flow of DEE from the engine will be delivered from a plenum downstream of the engine muffler to the exposure rooms through a heated stainless-steel deliver line. The required flow of DEE will be siphoned from this delivery line by a pressurized-air-controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose only exposure unit. All DEE exhaust will be routed to the building exhaust.

XIV. TEST SYSTEM SPECIFICATIONS

Species:	Mus musculus
Strain:	TBD (VAF)
Source:	Charles River Laboratories Raleigh, NC
Age at Arrive:	4-5 weeks
Age at Study Start:	6-7 weeks
Total Number of Animals:	275 males; 185 females (includes 55M/55F Big Blue mice)
Exposure Tube Acclimation Period:	~5 days
Identification:	Tail tattoo

XV. REASON FOR SELECTION

The ____ mouse was selected for this study on the basis of data available in the literature and a 13-week pilot study.

XVI. RANDOMIZATION

The mice which adapt to the exposure tubes will be randomly assigned to exposure groups by using body weight as a blocking variable to ensure that there are no statistically significant differences in initial group mean body weights. The weight distribution range (by sex) of the mice selected for the study will be as narrow as possible. A sufficient quantity of mice will be ordered and adapted to the exposure restraint tubes to ensure that no mouse will be selected whose body weight exceeds ±20% of the mean (by sex) at the start of the study. The Xybion Path/Tox System (Xybion Medical Systems Corporation, Cedar Knolls, NJ) will be used for randomization.

XVII. HOUSING AND MAINTENANCE

Mice shipping crates will be examined upon arrival for evidence of conditions likely to permit exposure to pathogens (soiled, wet, or otherwise damaged). The uncrating will be conducted at the door of the animal room. While being removed from the crates, the mice will be examined for evidence of shipping stress.

The mice will be acclimated in Room ____ of the LSL-II Building for 2 weeks prior to the start of exposure. Mice will remain on quarantine status until health screen procedures are completed, approximately 3 weeks after their arrival.

Throughout the study, cage space will meet the requirements stated in the 85-23 (1985) NIH "Guide for Care and Use of Laboratory Animals". The BNW animal care and use program, including the facility used for this study, is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

The mice will be housed approximately 5 mice per cage in hanging wire cages prior to identification and randomization at which time they will be housed individually in stainless steel wire cage units on stainless steel racks. Mice will be individually housed for the remainder of the study.

Room temperatures during the acclimatization and study will be maintained at 75 ± 2°F and relative humidities at 55 ± 15%. Measurements will be recorded at ~1 hour intervals.

Twelve hours light and twelve hours dark will be maintained with light starting at 0600.

The clinical veterinarian will make a visual inspection of the mice to be used in the study just prior to their release for the study. Mice will be released for study if the health screen doesn't provide evidence of pathogens or disease. Five mice per sex that were not used for the exposures will be reevaluated for evidence of disease 18-21 days after arrival at BNW. Sera will be tested for antibodies to Sendai virus, mouse hepatitis virus, GDVII virus, minute virus of mice, pneumonia virus of mice (PVM) and *Mycoplasma pulmonis*.

Sentinel mice (5M,5F) will be selected from the extra mice prior to the start of exposures. Serological evaluations will be performed on these mice at the time of terminal sacrifice of the core study mice.

During the acclimation period, approximately 7-10 days prior to the start of exposure, mice will be placed in nose-only exposure restraint tubes for increasing time up to 6 hours daily to allow them to adapt to confinement in the tubes. The mice which adapt to the restraint tubes will then be weighed, randomized, assigned to exposure concentration groups and identified individually with a unique number tail tattoo approximately two days before the exposure period.

Mice not used for the study will be euthanized one week after the start of the study. The disposition of these mice will be recorded on the Animal Disposition Record and retained in the study files.

Mice which die during the first week of the study will be replaced from the pool of unused mice.

XVIII. DIET AND WATER

NIH-07 Open Formula diet will be provided *ad libitum* except during the inhalation exposures when mice are in restraint tubes.

This diet is analyzed for contaminants and some nutrient components; these analyses are reviewed for acceptability using established standards prior to use of the food. There are no known contaminants in the diet which could be expected to alter the outcome of the study.

Fresh softened water (ion exchange softener, Illinois Water Treatment Company, Model 2R-2240, Rockford, IL) will be supplied *ad libitum* except during exposures. An automatic watering system (Edstrom Industries, Waterford, WI) will be used during the quarantine/acclimatization period and throughout the study. Water bottles will also be provided for the first three days after arrival.

Representative samples of animal drinking water from the LSL-II facility will be analyzed for contaminants at least once per year. There are no known contaminants in the drinking water which

could be expected to alter the outcome of the study. The hardness of the water will be checked approximately once every week. Records will be retained in the LSL-II Building Engineer's office.

XIX. ENVIRONMENTAL MONITORING

A. Air Supply

Room and exposure supply air will be HEPA-filtered. Both room and exposure exhaust air will pass through a series of two HEPA filters.

B. Temperatures

Temperatures in each exposure unit cabinet and in the exposure air stream at a nose port will be monitored at ~2-minute intervals and recorded at ~60 minute intervals during the daily exposure period. If the temperature is not within the acceptable range 75 ± 4°F, the system will alarm. Daily means for the exposure cabinet and the exposure atmosphere will be maintained within 75 ± 4°F.

Temperatures in the animal room will be monitored at ~2-minute intervals and recorded once every hour. If the temperature is not within the acceptable range 75 ± 4°F, the system will alarm. The acceptable range for individual animal room measurements and the daily mean will be 75 ± 4°F.

C. Relative Humidity

Relative humidity will be monitored at a nose-port in each exposure unit and recorded at ~60 minute intervals during the exposure period. If the relative humidity is not within the acceptable range 55±15%, the system will alarm. Daily means for the exposure unit will be maintained within 55±15%.

Animal room humidity will be monitored and recorded once every hour. If the relative humidity is not within the acceptable range 55±15%, the system will alarm. The acceptable daily mean is 55±15% relative humidity.

D. Airflow

Airflow will be monitored at an inlet and exhaust orifices using a calibrated photohelic pressure gauge. Both flows will be recorded once per hour during the exposure period. The acceptable minimum inlet flow is 250 ml/min per occupied exposure port.

Measurements out of specified limits will be recorded and result in an alarm so that the operator can take appropriate actions.

XX. EFFLUENT TREATMENT

Exposure unit exhaust will be passed through a series of two HEPA filters and a catalytic converter to remove all test article. The concentration of test article in the building exhaust will be measured once during the study to determine the efficiency of the effluent treatment system.

XXI. EXPERIMENTAL DESIGN

A. Route of Administration and Reason for Selection

The test articles will be administered by nose-only inhalation at concentrations of 1 and 10 mg/m^3 of RASS and 3 and 10 mg/m^3 of DEE. A filtered air control group will be included. The concentration of RASS and the route of administration used in this study were selected on the basis of route of human exposure and to provide target particle concentrations which exceed those determined for extreme human exposure to environmental tobacco smoke by a factor of approximately 10. The DEE concentrations were selected on the basis of data in the literature which indicate that these concentrations should result in a measurable carcinogenic response in the mouse.

B. Experimental Design

Group Number	Exposure Concentration (mg/m^3)	Core Study Mice[a,b,c]	Blue Mouse Micronucleus Assay Mice	Enzyme Assay Mice	Adducts Assay Mice	Plumonary Clearance Mice	Total
1	0	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
2	1 (RASS)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
3	10 (RASS)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
4	3 (DEE)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
5	10 (DEE)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
Sentinels	0	5M 5F	– –	– –	5M 5F	– –	5M 5F

[a]Includes 5 mice which will be used for a 13 week recovery period.
[b]Blood will be collected from 5/sex/group for carboxyhemoglobin determination.
[c]Urine will be collected from 5/sex from controls and RASS treated mice for nicotine and cotinine determination.

C. Animal Identification

Group Number	Exposure Concentration (mg/m^3)	Core Study Mice	Blue Mouse Micronucleus Assay Mice	Enzyme Assay Mice	Adducts Assay Mice	Plumonary Clearance Mice
1	0	1-15 (M) 101-115 (F)	16-25 (M) 116-125 (F)	26-31 (M) 26-31 (F)	32-36 (M) 32-36 (F)	37-54 (M) 137-154 (F)
2	1 (RASS)	201-215 (M) 301-315 (F)	216-225 (M) 316-325 (F)	226-231 (M) 326-331 (F)	232-236 (M) 332-336 (F)	237-254 (M) 337-354 (F)
3	10 (RASS)	401-415 (M) 501-515 (F)	416-425 (M) 516-525 (F)	426-431 (M) 526-531 (F)	432-436 (M) 532-536 (F)	437-454 (M) 537-554 (F)
4	3 (DEE)	601-615 (M) 701-715 (F)	616-625 (M) 716-725 (F)	626-631 (M) 726-731 (F)	632-636 (M) 732-736 (F)	637-654 (M) 737-754 (F)
5	10 (DEE)	801-815 (M) 901-915 (F)	816-825 (M) 916-925 (F)	826-831 (M) 926-931 (F)	832-836 (M) 932-936 (F)	837-854 (M) 937-954 (F)
Sentinels	0	SM1-SM5 SF1-SF5	– –	– –	– –	– –

D. Route of Administration

Nose-only inhalation

E. Frequency/Duration

Six hours/day plus T_{90}; 5 days/week; at least 13 weeks, excluding holidays and weekends

F. Daily Observations

Twice daily for moribundity and mortality on all surviving mice.

G. Clinical Signs

Weekly for all survivors and prior to unscheduled sacrifice for moribund or humane sacrifice mice.

H. Body Weights

Weekly on all surviving mice.

I. Urine Nicotine/Continine

During week 4 for 5/sex/RASS treated and filtered air control groups selected at random from the core study animals. Mice will be placed in metabolism cages for a 16 hour urine collection.

J. Micronucleus and Big Blue™ Mouse Assay

During week 5, the 10 Big Blue mice/sex/group will be anesthetized and the lungs perfused and removed aseptically. One-half of each lung will be used for the lung fibroblast micronucleus assay and the other half will be used for the Big Blue Mouse in vivo

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mutagenic assay. Slides or plates from 5 each from the 10 mg/m^3 RASS and DEE treated groups and the filtered air control group will be analyzed. If a high dose treated group(s) exhibits a positive response, then the slides and/or plates for the low dose group(s) will be scored.

K. Enzyme Activity

During week 4, lung, liver, and kidney samples will be collected from 6 mice/sex/group. Microsomal and cytosolic subcellular fractions will be prepared by differential centrifugation. Cytochrome P450 and glutathione-S-transferase activity will be determined.

L. Protein/DNA Adducts

Blood will be collected from 5 mice/sex/group from the adducts assay special study animals prior to terminal sacrifice. The mice will be placed in metabolism cages for a 16 hour urine collection. At terminal sacrifice samples of the lung and trachea will be collected from these animals and frozen at -80°C. The urine and tissue samples will be evaluated for protein/DNA adducts.

M. Carboxyhemoglobin

Blood will be collected from 3/sex/group selected at random from the core study animals during weeks 5, 9, and 13. Mice will be removed from the exposure chamber within one hour before the end of the daily exposure period, anesthetized with ~70% CO_2, and bled from the supra-orbital sinus.

K. Clinical Pathology Evaluations

Prior to terminal necropsy 10 mice/sex/group selected at random from the core study animals will be anesthetized with ~70% CO_2 and blood samples will be collected from the supra-orbital area into tubes containing potassium EDTA for a leukocyte differential count.

L. Lung Clearance

On the day following the termination of the 13-week RASS and DEE exposures, 18 male mice/group will be exposed to 10 mg TiO_2/m^3 for 5 consecutive days. Lung burdens of TiO_2 will be determined, using ICP-AES techniques, on 3 animals/group at 0, 1, 3, 9, 30, and 90 days following termination of TiO_2 exposures.

M. Minute Volume

Six male mice per group (one group/day) during the TiO_2 exposures. Collection of respiratory data from the plethysmographs will be conducted between 1 hour after the start of exposure and the end of the exposure period.

Tidal volume (ml) and respiration rate (breaths/min) will be monitored continuously for 10 minutes. Average tidal volume and respiration rates will be calculated and stored for all breaths occurring during each 10-second interval of monitoring. Minute volume (ml/min) will be calculated as the product of the 10-second average tidal volume and the 10-second average respiration rate. The mean and standard deviation of the minute volume for the entire 10-minute recording session will then be calculated from the 10-second average minute volumes.

Body weights will be recorded on these mice on the day that the minute volume data are collected. If minute volume data are not collected on a scheduled weighing day, these weights will be in addition to the regular weekly body weights.

N. Proliferating Cell Nuclear Antigen

At histopathology, additional sections of nose, larynx, and lungs will be cut. Mucosal epithelial cell replication rates in these tissues will be compared by labeling the nuclei with the PC-10 monoclonal antibody and counting labeled nuclei.

XXII. POSTMORTEM OBSERVATIONS AND MEASUREMENTS

A. Necropsy

A complete necropsy will be performed on all core study mice. Findings will be recorded on an Individual Animal Necropsy Record (IANR) form. All 13-week study mice will be necropsied on the day following the last day of exposure. The recovery group will be necropsied 13 weeks after the last exposure. Mice will be killed by anesthetization with CO_2 followed by exsanguination. Necropsies will include an external examination of all body orifices, an examination of tissues/organs, and fixation in 10% neutral buffered formalin (NBF) of the following tissues:

Adrenals
Brain (medulla/pons, cerebellar cortex, cerebral cortex)
Cecum
Colon
Duodenum
Esophagus
Eyes
Femur (including joint)
Gallbladder
Harderian glands
Heart/aorta
Ileum
Jejunum
Kidneys
Liver
Lungs (trachea, larynx, tongue, pharynx)
Lymph nodes
Mammary gland/skin
Nose
Ovaries
Pancreas
Pituitary
Preputial/clitoral glands
Prostate
Rectum
Salivary glands
Sciatic nerve
Seminal vesicles/coagulation glands
Spinal cord (cervical, thoracic, lumbar)
Spleen
Sternum with bone marrow
Stomach
Testes/epididymis
Thigh muscle
Thymus
Thyroid (including parathyroids)
Tissue masses/tumors
Urinary bladder
Uterus
Zymbal's gland

B. Organ Weights

Organ weights will be recorded from all core study mice for both adrenal glands (0.001 g), brain (0.01 g), epididymides (0.001 g), heart (0.01g), kidneys (0.01g), liver (0.01 g), lung with trachea (0.01 g), spleen (0.01 g), and testes (0.01 g). Organ to body weight and organ to brain weight ratios will be calculated. Organ weights from moribund/humane sacrifices will be collected and reported, but not included in the data analysis.

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C. Histopathology

Organs/tissues collected from the filtered air control and the high concentration RASS and DEE groups will be processed to slides and stained with hematoxylin and eosin for histopathologic examination. Special stains will be used at the discretion of the pathologist. Data will be entered onto the Xybion Path/Tox System for all mice.

A complete histopathologic evaluation inclusive of gross lesions will be performed on all core study mice from the filtered air control and the high concentration RASS and DEE groups sacrificed at the end of the 13 week exposure period. Histopathologic evaluation will be conducted on gross lesions and designated tissues from all core study animals. Tissues exhibiting effects in the high concentration group(s) will be examined in the low concentration group(s) and in the animals sacrificed at the end of the recovery period. Slides may be peer reviewed by the Sponsor. A complete histopathologic evaluation will be conducted on the following tissues :

Accessory genital organs (prostate, seminal vesicles, coagulation glands)
Adrenals, right and left
Aorta (throacic)
Brain (cerebral cortex, cerebellar cortex, pons/ medulla oblongata)
Caecum
Clitoral glands
Colon
Duodenum
Epididymis
Eyes with optic nerve (if grossly abnormal)
Femur, including joint
Harderian glands
Heart
Ileum
Jejunum
Kidney, right and left
Larynx/laryngopharynx (step sections, 4 levels, laryngopharynx at the base of epiglottis)
Liver, gallbladder
Lungs (6-μm sections every 5 mm)
Lymph nodes (mandibular, mesenteric, bronchial and mediastinal)
Mammary glands
Musculature (thigh)
Nose/nasopharynx (4 levels; Young, 1981)
Oral cavity
Ovaries (mesovaries), right and left
Pancreas
Parathyroid glands
Peripheral nerve (sciatic)
Pituitary gland
Preputial glands
Rectum
Salivary glands (submandibular and sublingual)
Skin
Spinal cord (cervical, thoracic, and lumbar)
Spleen
Sternum with bone marrow
Stomach (forestomach and glandular stomach)
Testes, right and left
Thymus
Thyroid glands
Tongue
Trachea
Urinary bladder
Uterus
Zymbal's glands
All grossly visible tumors or lesions suspected being tumors

D. Statistical Analysis

Results from this study will include incidence tables of clinical signs, gross lesions, and histopathological observations. Group means will be calculated for:

Body weight
Body weight gain
Organ weight
Organ:body weight ratio
Organ:brain weight ratio
Leukocyte parameters
Nicotine and cotinine levels
Lung clearance
Incidence of micronuclei
Incidence of mutant colonies
Enzyme activity

Any group with a sample size less than six will have only means and standard deviations calculated. Group variances for body weight, clinical pathology and organ weight data will be compared using Bartlett's test. When the differences between group variances are not significant ($p>0.01$), a one-way analysis of variance (ANOVA) will be performed. If significant differences ($P<0.01$) among the means are indicated by the ANOVA, Dunnett's multiple comparison test will be used to determine the intergroup differences. Dunnett's makes pairwise comparisons among all group means including the control groups. Significance will be declared at the 0.05 and 0.01 alpha levels.

In the event that Bartlett's test indicates significant differences between group variances for a given parameter, the mean values between the control and each concentration group will be compared using the Behrens-Fisher t-test (Satterthwaite's method will be used to adjust for degrees of freedom). Significance will be declared at the 0.05 and 0.01 alpha levels if the P value is less than alpha divided by the number of comparisons made (Bonferroni's adjustment for multiple comparisons; Miller 1981).

The percent difference in mean body weights (MBW) between treated and control animals will be calculated as follows:[1]

$$\frac{\text{MBW of Exposed Group - MBW of Control Group}}{\text{MBW of Control Group}} \times 100\%$$

E. Study Conduct and Records Retention

Documentation will be maintained in such a manner that confidentiality for the Sponsor, in all aspects of the study conduct, will be assured.

This protocol will be the controlling document. The Study Director is to be notified immediately for clarification if discrepancies occur between the protocol and the SOPs. Any changes to this protocol will be made in the following manner. If BNW initiates the change, not associated with cost, verbal (telephonic) approval will be obtained from the Sponsor followed by written documentation by BNW to the Sponsor. Any change which impacts the cost or schedule of the study will be documented in writing and approved by the Sponsor Representative's signature prior to implementation. The Sponsor may initiate modification to the protocol by telephonic authorization to BNW followed by written documentation as stated above. This study will be conducted in compliance with Good Laboratory Practice

[1]This procedure is currently in use by the National Toxicology Program, NIEHS, Research Triangle Park, NC.

regulations, 21 CFR 58. All records required to reconstruct the study will be maintained as stipulated in 21 CFR 58.190. All paraffin blocks and wet tissues resulting from any portion of this study will be retained by BNW until 6 months after acceptance of the final report at which time they will be sent to the Sponsor. If requested by the sponsor, all microscopic slides will be submitted to the Sponsor with the final draft report. Records to be retained in study archives will be specified by the SOP entitled "Records to be Retained for Philip Morris Studies". Records that accumulate during the study will be retained at BNW until they are shipped to the Sponsor immediately prior to the termination of the contract or until the Sponsor requests transfer, whichever occurs first.

F. Draft Report

Following a Quality Assurance audit of the raw data and data tables, a complete and detailed draft report will be submitted to the Sponsor within the time frame of the contract.

G. Reference

Miller, R.G. Jr. 1981. *Simultaneous Statistical Inference.* Springer-Verlag, New York, NY.

XXIII. BNW APPROVAL

Earl W. Morgan, DVM, ACVPM, DABT
Principal Investigator

Date

R.A. Gelman, MS
Quality Assurance Auditor

Date

XXIV. SPONSOR APPROVAL

George J. Patskan, PhD
Sponsor's Representative

Date